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Intraluminal Epidermal Growth Factor Affects Growth of N-Methyl-N-nitrosourea-initiated Rat Bladder Carcinoma

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To confirm our recent finding that epidermal growth factor (EGF) appeared to contribute to the tumor-enhancing effect demonstrated by normal rat urine, we conducted 2 experiments using our heterotopically transplanted rat urinary bladder model. In experiment 1, after a single dose (0.25 mg) of N-methyl-N-nitrosourea (MNU), we intravesically administered EGF (0.5 ml of 500 ng/ml phosphate-buffered saline) once a week for 30 weeks. Instillation of EGF induced a significantly larger number of tumors than did instillation of the vehicle ($P=0.03$). EGF without MNU initiation did not induce tumors. In experiment 2, 2 groups received instillation of killed *Escherichia coli* (5×10^8 cells)/0.5 ml phosphate-buffered saline once a week for 4 weeks to expand the MNU-initiated cell population. Subsequent EGF treatment significantly increased the incidence of tumors ($P=0.01$). In the groups which did not receive killed *E. coli*, EGF treatment induced a significantly higher number of tumors than did vehicle treatment ($P<0.001$). All of the tumors were low-grade, superficial transitional cell carcinomas. These observations indicate that EGF acts as a growth-stimulating factor on dormant neoplastic cells and thereby increases the number of tumors.

Key words: Epidermal growth factor — Bladder tumor — Growth stimulation — N-Methyl-N-nitrosourea

A majority of urinary bladder cancers are superficial and of low grade, but new tumor(s) appear after endoscopic resection of the original tumor in approximately 70% to 80% of patients.[1)] By using the HTB[4] system, an *in vivo* model, which was developed by us, we have demonstrated that growth factors present in the urine play a significant role in the development of carcinoma initiated by MNU,[2,3)] and that EGF appears to be an important component of the tumor-enhancing urine fractions.[3,4)] Separately, we have shown that EGF (or signal transduction mediated by EGF receptor) acts as a mitogen on several rat bladder carcinoma cell lines *in vitro*, but does not enhance a malignant phenotype.[5–7)] In the study in which EGF demonstrated an enhancement of MNU-initiated carcinogenesis,[3)] the EGF used was derived by chromatographic separation of urine collected from normal rats. Thus, a possible effect of one or more other urinary components that may have existed in the EGF-rich preparation could not be ruled out. The current experiment was an attempt to demonstrate unequivocally the role of EGF in urinary bladder carcinogenesis *in vivo*. We used purified authentic rat EGF. Its effect was tested under two slightly different experimental conditions. In experiment 1, EGF treatment was started 1 week after a single low dose of MNU and was continued until the end of the experiment. In experiment 2, a short-term KEC treatment was inserted between MNU initiation and EGF treatment in an attempt to expand the MNU-initiated cell population.[8)]

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[4] The abbreviations used are: HTB, heterotopically transplanted rat urinary bladder; MNU, N-methyl-N-nitrosourea; EGF, epidermal growth factor; KEC, killed *Escherichia coli*; PBS, phosphate-buffered 0.5% NaCl solution.

MATERIALS AND METHODS

Animals A total of 400 young male Fischer 344 rats (160–190 g body weight, Harlan Sprague-Dawley Inc., Indianapolis, IN) were housed in plastic boxes, 4 or 5 per cage, in an air-conditioned room at 22°C with 50% humidity under a 12-h light-dark cycle. They had free access to a pellet diet (Purina 5012; Ralston Purina Co., St. Louis, MO) and tap water.

HTB system We used the HTB system, which we had developed in our laboratory, for investigating the role of urine in bladder carcinogenesis.[2)] In brief, a bladder taken aseptically from a donor rat was connected to a reservoir through silastic tubing. The bladder-reservoir unit was then transplanted into a syngeneic recipient in such a way that the bladder portion was placed within the gluteal muscle and the reservoir portion in the dorsal subcutaneous tissue. Four weeks after transplantation of the urinary bladder, the system is ready for experiment. Thus, one half of the rats purchased were used as bladder donors.

EGF Purified rat EGF (Harlan Bioproducts for Science, Indianapolis, IN) derived from the submaxillary gland of male rats was dissolved in PBS at a concentration of 500 ng/ml and stored at –20°C in portions sufficient for single use. Once the suspension was thawed, any portion remaining after use was discarded.

Retention time of EGF instilled into the HTB To determine how much EGF should be used for intravesical instillation, we conducted two preliminary experiments. The first was designed to determine the EGF level in normal rat urine. Urine was collected between 10 AM and noon from 4 young male Fischer 344 rats by puncture of the urinary bladder exposed by laparotomy, and was analyzed for EGF by radio-immunoassay (Biomedical Technologies Inc., Stoughton, MA). In a second experiment, we determined how long EGF remained in the lumen after a single intravesical instillation. Six rats with HTBs were used. One half ml of PBS containing 500 ng/ml of EGF [approximately twice the concentration in normal rat urine (see "Results")] was instilled into the HTBs. The bladder content was aspirated as completely as possible from each of 2 rats immediately, and 48 h and 4 days later. We made no attempt to remove EGF completely by repeated washings of the bladder lumen. In 5 of the 6 rats, the same dose of EGF was instilled again after washing of the HTBs twice with PBS, and 7 days later the bladder content was withdrawn as completely as possible. The aspirates from HTBs were analyzed for EGF by radio-immunoassay.

Suspension of KEC *E. coli* (strain 3921) provided by Dr. John R. Warren of Northwestern University Medical School was used. The microorganism, grown overnight in Luria-Bertani medium, was heat-killed in boiling water for 30 min. The suspension was centrifuged, and the sediment was suspended in PBS at a density of 1×10^9 cells/ml, divided into multiple aliquots, and stored at −20°C. Once the suspension was thawed, any portion remaining after use was discarded.

Experimental design Two experiments were conducted.

Experiment 1: Four weeks after the establishment of the HTB system, 90 rats were divided randomly into 3 groups receiving into their HTBs a single dose of 0.25 mg of MNU freshly dissolved in 0.5 ml of 0.9% NaCl (groups 1 and 2) or vehicle only (group 3) (Fig. 1A). In groups 1 and 3, EGF (0.5 ml of 500 ng/ml EGF in PBS) administration into the HTBs once a week was started 1 week after MNU initiation and continued for the entire duration of the experiment (30 weeks). Group 2 received PBS treatment for 30 weeks, beginning 1 week after MNU initiation.

Experiment 2: One week after initiation with 0.25 mg of MNU, 96 rats were divided into 4 groups (Fig. 1B). Rats in groups 1 and 2 each received into their HTBs KEC (5×10^8 cells suspended in 0.5 ml of PBS) once a week for 4 weeks, and rats in groups 3 and 4 received PBS during the same period. The subsequent 2 weeks were a period of recovery from the KEC-induced inflammation; 1 week after the last KEC instillation, the bladder aspirate was examined for the number of polymorphonuclear leukocytes, which is a sensitive measure of inflammation induced by KEC treatment.[9] After aspiration of the preexisting fluid, the HTB was washed once

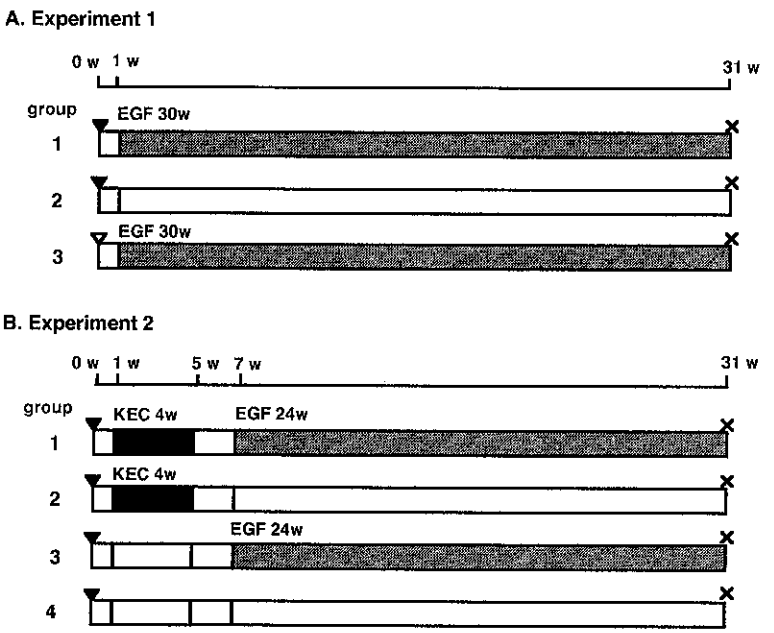

Fig. 1. Experimental design. Four weeks after establishment of the HTB system, rats were divided into groups that were treated with a single intravesical dose of 0.25 mg of MNU in 0.9% NaCl solution (▼) or vehicle (▽). In experiment 1, rats were divided into 3 groups which received EGF or vehicle (PBS) once a week for 30 weeks. For experiment 2, the first 2 groups received a 0.5 ml suspension of KEC once a week for 4 weeks in an attempt to expand the dormant tumor cell population (see references 7 and 15). EGF (or vehicle) treatment was begun 2 weeks later and was continued for 24 weeks.

with 1 ml of PBS. After having confirmed that the leukocyte count had returned to the pre-KEC-treatment level in 2 weeks, we began weekly instillation of EGF (0.5 ml of 500 ng/ml EGF in PBS) (groups 1 and 3) or PBS (groups 2 and 4) and continued this for 24 weeks, until the end of the experiment.

In both studies, at the end of the experiment aspirates from all HTBs were cultured to evaluate bacterial growth. Animals whose aspirate culture was positive were removed from the study. After completion of the experiments, HTBs were removed and opened by a longitudinal incision, stretched over a piece of cardboard, and fixed in 10% neutral formalin. After fixation overnight at room temperature, the mucosa was inspected for the presence of tumors. The number, size, and location of tumors were recorded. Tumor size was calculated from the measurement of the longest and shortest axes, with the assumption that a tumor is an elliptical mass. The bladders were cut into multiple strips in such a way that all gross lesions could be examined microscopically. Urothelial lesions, grade and stage of tumors, and type of epithelial differentiation were classified according to the criteria previously described.[10]

Statistical analysis Tumor incidence and tumor volume were compared between groups with Fisher's exact test. The total number of tumors was compared between groups by using the chi-square test with one degree of freedom. This test compared observed and expected numbers of tumors. For all tests, $P<0.05$ was taken as the criterion of statistical significance.

RESULTS

Retention time of instilled EGF in the HTBs The EGF concentration in urine collected from normal young adult male rats ranged from 198 to 323 ng/ml (254±64 ng/ml). The EGF content in the aspirates after instillation of 0.5 ml of 500 ng/ml of EGF is shown in Fig. 2. There was an apparent reduction in EGF content to 150 ng in the aspirate drawn immediately after EGF instillation. This, we believe, was largely due to incomplete withdrawal of bladder contents. Subsequently, however, loss of EGF was gradual, and the aspirates 7 days after instillation contained 102±26 ng of EGF/0.5 ml aspirate (mean±SD, N=5). The results indicated that at least 40% of the instilled EGF remained in the lumen on day 7. The concentration of EGF on day 7 was slightly lower than that of normal urine of male Fischer 344 rats.

Experiment 1 There was no difference in body weight among groups. Nine rats were removed from the study; these included 5 rats with culture-proved infection of their HTBs and 4 rats with the closure of the reservoir-bladder communication which made delivery of test material impossible. Thus, our analysis was based on 81 rats.

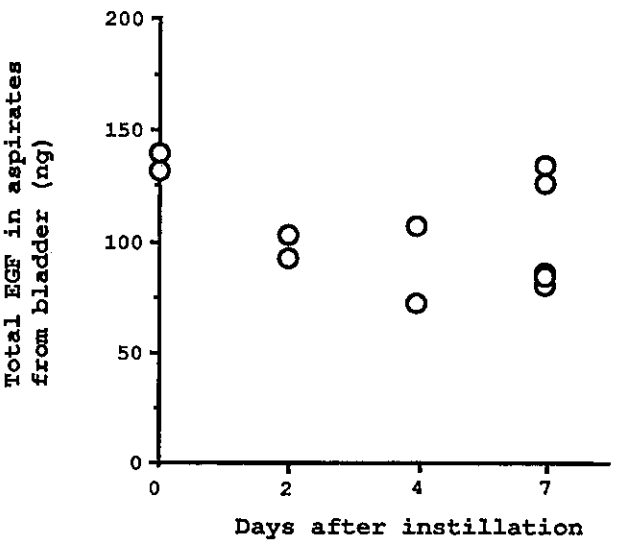

Fig. 2. Retention of EGF instilled into the HTB. To determine how long EGF may remain in the lumen after a single intravesical instillation, we instilled 250 ng of EGF in 0.5 ml of PBS into HTBs, and the bladder content was aspirated as completely as possible at the indicated time for determination of the residual EGF. Each circle represents 1 animal.

The incidence and number of tumors are presented in Table IA. Continuous instillation of EGF into the bladders without MNU pretreatment did not induce tumors. Microscopic examination was negative except in 1 bladder showing simple urothelial hyperplasia. Although no significant difference in tumor incidence was observed between groups 1 and 2 (21% and 12%, respectively), a significant increase in the total number of tumors upon EGF treatment was demonstrated ($P=0.03$). The tumors in group 1 ranged from 0.4 mm^3 to 60 mm^3 in volume, whereas the tumors in group 2 ranged from 0.4 mm^3 to 3.1 mm^3. All tumors were non-invasive transitional cell carcinomas that were classified as grade 1 in 14 cases and grade 2 in one case.

Experiment 2 There was no difference in body weight among groups. Fourteen rats were removed from the study; they included 6 rats with infected HTBs, 5 rats with closure of the reservoir-connector system, and 1 rat each from groups 2, 3, and 4 which were found dead of unknown causes during the 11th, 12th, and 11th weeks of the experiment, respectively. Thus, our analysis was based on 82 rats (Table IB). The maximal tumor-enhancing effect was demonstrated in group 1 (MNU followed by KEC/EGF). When group 1 was compared with group 2 (MNU followed by KEC/PBS), a significant increase in tumor incidence ($P=0.01$) was observed. When group 3 (MNU followed by EGF) was compared with group 4 (MNU followed by PBS), the

Table I. Incidence and Number of Tumors

A. Experiment 1

Group	No. of rats	Treatment		Rats with tumors (%)	Total no. of tumors/group
		MNU	EGF/PBS		
1	28	+	EGF	6 (21)	12[a]
2	26	+	PBS	3 (12)	3
3	27	−	EGF	0 (0)	0

a) $P=0.03$ compared to group 2.

B. Experiment 2

Group	No. of rats	Treatment			Rats with tumors (%)	Total no. of tumors/group
		MNU	KEC	EGF/PBS		
1	23	+	+	EGF	14 (61)[a]	20
2	18	+	+	PBS	3 (17)	7
3	20	+	−	EGF	7 (35)[b]	9[c]
4	21	+	−	PBS	1 (5)	1

a) $P=0.01$ compared to group 2.
b) $P=0.02$ compared to group 4.
c) $P<0.001$ compared to group 4.

Table II. Distribution of Tumors by Size

Group	Treatment		Tumor size (mm^3)				Total no. of tumors
	MNU	EGF (weeks)	<0.9	1–9.9	10–99	>100	
Experiment 1							
1	+	+ (30)	5	3	4	0	12
2	+	−	2	1	0	0	3
Experiment 2							
3	+	+ (24)	2	5	1	1	9
4	+	−	1	0	0	0	0

EGF treatment effectively enhanced the total number as well as the incidence of tumors ($P<0.001$ and $P=0.02$, respectively). All tumors were non-invasive transitional cell carcinomas, mainly of grade 1 and occasionally of grade 2. Two tumors in group 1 and 4 tumors in group 3 showed squamoid and/or squamous differentiation.

Distribution of tumor by size Table II shows the distribution of tumors by size. Although statistical significance was not reached ($P=0.17$), probably because of the small number of tumors in the control groups, there was a clear trend of tumors having a larger size in EGF-treated groups; almost all tumors observed in the groups receiving only the vehicle after MNU initiation in both experiments were minute nodules of less than 0.9 mm^3. In the bladders treated with EGF, 14 of 21 tumors (experiments 1 and 2 combined) were larger than 1 mm^3 in volume, and the largest observed were 60 mm^3 and 400 mm^3 in experiments 1 and 2, respectively.

DISCUSSION

Because EGF is known to be excreted in large quantities in urine,[11] and because its receptors have been identified in bladder cancers,[12, 13] a role of EGF in the development of bladder cancer has been suggested.[14] Using the HTB system developed by us, we have demonstrated that growth factors present in urine play a significant role in the development of low-grade superficial carcinomas initiated by MNU, and our data suggested that EGF was an important component of the tumor-enhancing urine fractions.[3, 4] A growth-stimulatory effect of EGF was also demonstrated *in vitro* with rat bladder tumor cells.[5–7]

Our primary objective in this study was to demonstrate that EGF is indeed a urine-borne growth factor that, by itself, is capable of enhancing MNU-initiated urinary bladder carcinogenesis. We used purified rat EGF at a

concentration comparable to that in urine. In experiment 1, weekly injection of 500 ng/ml of EGF, which was started 1 week after MNU initiation and continued for 30 weeks, induced a significantly greater number of tumors compared with the injection of vehicle ($P=0.03$). As we anticipated, EGF did not induce tumors in the group that was not pretreated with MNU. Because EGF treatment was started 1 week after MNU administration, intraluminal EGF should readily have reached the basal layer of the urothelium damaged by MNU.[15] Thus, EGF-induced urothelial proliferation might have "fixed" unrepaired promutagenic DNA damage caused by MNU. This was a possible effect because, in a study by Kadlubar *et al.*, repair of MNU-induced O^6-methylguanine in the rat bladder epithelium continued during 21 days after exposure.[16]

Experiment 2 had 2 objectives. The first was to rule out a role of EGF in "fixing" promutagenic DNA damage by MNU. The second objective was to test the effect of EGF on the dormant neoplastic cell population that was expanded by a brief exposure to KEC, the effect of which was demonstrated in one of our recent studies.[8] To attain the first objective, we began EGF treatment 7 weeks after MNU initiation. A significantly greater number of tumors was observed in the EGF-treated group (group 3) than in the vehicle-treated group (group 4) ($P=0.02$). The enhancing effect of EGF was also shown by a significantly higher incidence in the MNU/KEC-treated group than in the control group (group 1 versus group 2, $P=0.01$).

All of the tumors observed in the present investigation were non-invasive carcinomas, mainly of grade 1 and occasionally of grade 2. There was no evidence that EGF treatment altered the invasiveness of the tumors.

What is the mechanism by which EGF exerts tumor-enhancing activity? It is reasonable to assume that EGF action is mediated by EGF binding to its receptors distributed on the surface of urothelial cells. In the normal urothelium EGF receptors are localized at the basal cell layer in human[12] and rat (Wu and Oyasu, unpublished data), whereas in tumors, they are expressed in all layers[12] (Wu and Oyasu, unpublished data). These observations are in keeping with the marked increase in ^{3}H-thymidine labeling indices in tumors and hyperplastic urothelium, but not in normal urothelium, when tumor-bearing HTBs were challenged with a single dose of EGF.[3] Two mechanisms can be considered. First, EGF may act as a growth factor for the dormant neoplastic cells whose potential for autonomous growth is extremely limited. These cells can be stimulated by EGF to grow because they express EGF receptors. Second, by an unknown mechanism, EGF could convert MNU-initiated cells so that they acquire the potential for autonomous growth. As we reported recently,[17] a large number of genetically altered cells remain dormant for a long time after a single exposure to 0.25 mg of MNU. However, when a chronic inflammatory stimulus is applied, these cells acquire neoplastic potential and develop into a large number of tumors.[8,17] This explosive pattern of growth has not been observed in bladders treated with urine or with specific tumor-promoting urine fractions.[2,3] If the action of EGF were similar to that of an inflammatory stimulus, a far greater number of small tumors would develop. In the present study, the EGF treatment induced only 21 tumors in a total of 48 bladders that had been initiated with MNU (group 1 in experiment 1 and group 3 in experiment 2 combined, Table I). Although statistical significance could not be demonstrated ($P=0.17$), probably owing to the small number of tumors observed in the control group, many of the EGF-induced tumors were larger than those in the control group (Table II). Therefore, we believe that the main function of EGF is to stimulate the growth of dormant neoplastic cells, and that the stimulation results in an increase in the number of visible tumors and in a trend toward a larger size. Our data clearly demonstrated that intraluminal EGF at a physiologic concentration can enhance carcinogenesis in the MNU-initiated urothelium without any other urine components. Our observation suggests that urinary EGF, at least in part, accounts for the high rate of recurrence of superficial bladder tumors.

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